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(70) Proprietor: **PRUTEC LIMITED**  
**142 Holborn Bars**  
**London EC1N 2NH (GB)**

(72) Inventor: **Sutherland, Ranaid**  
**23, rue de la Filature**  
**CH-1227 Carouge (CH)**  
Inventor: **Daehne, Claus**  
**103 av. du Bois de la Chapelle**  
**CH-1213 Onex (CH)**  
Inventor: **Revillet, Georges**  
**20, chemin Francois Chavaz**  
**CH-1213 Onex (CH)**

(74) Representative: **Dousse, Blasco et al**  
**7, route de Drize**  
**CH-1227 Carouge/Genève (CH)**

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**EP 0 184 600 B1**

## Description

The present invention concerns a method for ascertaining at least one parameter of species in a liquid analyte, e.g. for determining one or more species dissolved therein.

5 This method relates to the known techniques in which an optical waveguide carrying a totally reflected light signal is contacted with an analyte and the evanescent wave component of said signal interacts with the analyte at the solid-liquid interface in a manner responsive to some parameters inherent to the species therein. For instance, information on such parameters may concern an absorption of part of the luminous energy of the incident signal at the points of reflection thereof within the waveguide by the molecules of the species of interest at said interface or to an excitation of some fluorophores by said luminous energy with the consecutive production of a fluorescence signal characteristic of such species. In general, the interaction is limited to the region corresponding to the depth of penetration into the analyte of the evanescent wave component of the incident light, this depth ranging from a few angströms to some hundreds of angströms starting from the waveguide surface.

15 Although it is generally known that the aforementioned interaction may provide information on some parameters in the bulk solution (Hardy, USP 4,050,895), recently published work has shown that improved results (i.e. better sensitivity and accuracy) are obtained when the effective interaction of the evanescent wave component with the analyte (or rather, the leaking into the analyte of a substantial portion of the energy thereof) is limited to involve a single layer (generally monomolecular) of compounds of interest bound to the surface of the waveguide. In other words, one has recently found that a very useful new analytical technique can be based on attaching to the waveguide, before contacting it with the analyte to be investigated, a reactant specific to a species of said analyte and, thereafter, immersing it into said analyte; in these conditions, the species of interest will bind to the said reactant and provide at the surface of the waveguide a layer of a complex whose content of the species of interest (i.e. the actual density of said species in the area of interaction) will grow very rapidly with time and provide increased interaction with the light travelling in the waveguide and stronger response at the output thereof (Kronick and Little, USP 3,939,350).

In such types of analysis involving the formation of a very thin layer of product of interest on the waveguide surface, the interaction of the light signal with the bulk of the solution is considered a nuisance (background noise) and attempts have been made to minimize it as much as possible. For instance, compromising condition between maximal interaction of the evanescent component with the monomolecular layer deposited on the waveguide surface and minimal interaction with the bulk solution can be achieved by controlling the depth of penetration of said evanescent component outside the material of the waveguide at the waveguide/analyte interface. Such control can be effected by selecting a waveguide with a suitable refractive index  $n_1$  relative to that  $n_2$  of the solution and by choosing adequately the angle of total reflection in the guide as well as the wavelength of the incident light (more detailed explanation on why this can be so and how to make such selections will be provided hereafter). For instance in co-pending application EP—A—75353, it has been disclosed that this depth of penetration can be optimized to match with the thickness of said layer of interest or to be in excess thereof.

40 However, it has now been found that unexpectedly this approach is not always the most desirable; indeed, it has been found with surprise that, under some conditions, the penetration of the evanescent wave component distinctively beyond the distance corresponding to the thickness of the layer of interest can be extremely useful in concurrently providing analytical results on parameters of the analyte other than that specifically involved in said layer of interest, e.g. on species in solution in the analyte. Therefore, this discovery led to the definition of one aspect of a new analytical method outlined in claim 1 given in annexe to this specification. Moreover, this method was also extended to further aspects after realizing that useful interactions of the light carried by the waveguide with the analyte may involve more than one specific area of the waveguide (i.e. the area where interaction takes place with either a single layer coating on this area or with the bulk solution or, simultaneously with both bulk and coating), i.e. may involve two or several distinctly different areas of said waveguide (see co-pending application EP—A—185126). For instance, in one area the interaction will take place with the bulk solution and in another or more optically separated areas the interaction will take place with one or several layers of interest. Annexed claim 1 actually summarizes the complete scope of this method of the invention.

In the method of the invention, any type of interaction of the light contained in the waveguide with the analyte can be considered. Thus, this interaction can result from absorption of part of the signal, the output response being then a decrease in output energy gathered by the collecting and detecting means located at the outlet of the waveguide. Alternatively, the interaction may result in the production of fluorescence if the species of interest (whether located in the bulk analyte or in a coating of interest at the waveguide surface) can generate fluorescence under excitation by the incident signal. Such is the case for instance in fluorescence type assays where one of the partners in a complex under formation on the surface of the waveguide comprises a fluorophore group which induces fluorescence upon formation of said complex. Otherwise, useful responses can also result from the scattering of the incident light by molecular aggregates built on the surface of the illuminated waveguide.

65 In order to practically carry out the method of the invention, one can for instance use a waveguide in the form of an optical fiber or a glass or plastic slide coated with a reagent specific to one first species

## EP 0 184 600 B1

dissolved in an analyte which additionally contains another or more species of interest. In an example to be developed in detail hereinafter, this first species can be a specific hemoglobin compound in a sample of blood also containing other hemoglobin or blood factors. Thus, in this case, the waveguide will carry, attached thereto, an antibody specific to that first species and, upon contacting the lit waveguide with the blood sample and arranging the measurement conditions (as specified hereafter) for providing an effective and signal generating interaction of the light in the waveguide with, simultaneously, the bulk solution and a monomolecular layer of a complex involving the antibody and that first species that forms on the waveguide surface, a signal is provided at the outlet of the guide, this signal being representative concurrently or simultaneously of the total hemoglobin (or other blood factors) having interacted in bulk with the excitation signal and of said first species involved in the formation of the complex.

In this case, the signal at the outlet of the waveguide is representative of two independent effects and can be decoded by simple means since the response to the bulk hemoglobin corresponds to an instantaneous partial extinction of the signal output collected from the waveguide end (this is actually the aforementioned background noise) while the response to the thin layer is a time dependent signal due to the formation of said complex layer of antibody and the first specific species to be determined which is a rate reaction.

Otherwise, if two specific factors in the analyte (for instance factor 1 and factor 2 among others present) must be determined, a waveguide with two independently working optical areas is preferably selected, each area being provided with one reagent (antibody) specific to one of said factors to be determined (see EP—A—185126 cited as cross-reference). In such case, the two response signals collected at the output of the waveguide (this being so if the waveguide is not provided already with separate outputs) are either (a) phase dependent or (b) frequency dependent.

Case (a) may be illustrated by a waveguide provided with two independent optical elements such as the two oppositely facing parallel walls of an analytical cuvette, said walls being light conductive for a fully reflected signal and each internally coated with one of the two aforementioned reactants, each being specific to one of the two factors (factor 1 and factor 2) to be determined in the analyte. In this case the two elements are illuminated in turn (alternatively applied pulses), the mode of application being also used for synchronization purposes at the detecting and processing end to ensure proper separation and independent display of the corresponding signals.

Case (b) may be illustrated by a waveguide structure comprising two physically separated areas on the same light path of the waveguide (i.e. not separated optically) but providing responses at two different waveguides (this can be implemented, for instance, by having one area responsive to absorption, e.g. at the excitation wavelength and another area providing a fluorescent response, i.e. a signal of a wavelength different from the excitation wavelength). In this case the detector unit is provided with means to separate the components of the output consisting of a signal with two wavelengths into individual signals by usual means (band-pass filters or dichroic beam splitters). Such a situation can result for instance from attaching to a first area of the waveguide a first reagent specific of factor No. 1 to be analyzed, the layer of reaction product being light absorbing, and attaching to a second area of the waveguide a second reagent specific of factor No. 2, the reaction product of said second reagent and factor No. 2 being fluorescent under excitation by the incident light.

Of course case (b) can also be illustrated by a variant of the structure of case (a), i.e. with one of the separately illuminated waveguide element being responsive to absorption while the other is fluorescence responsive.

The practical aspect of the invention will now be illustrated with reference to actual analysis cases. The first case refers to blood analysis and, more particularly, to the direct determination in blood samples of hemoglobin and various other hemoglobin factors such as glycosylated hemoglobin, this being, if desired, relative to total hemoglobin in this sample.

Glycosylated hemoglobins ( $\text{HbA}_{1c}$ ,  $\text{A}_{1b}$  and  $\text{A}_{1o}$ ) are important factors in the diagnosis and monitoring of patients with diabetes. The determination of the content of  $\text{HbA}_{1c}$  (which amounts to about 80% of total glycosylated hemoglobins ( $\text{HbA}_1$ )) relative to total hemoglobin (i.e.  $\text{HbA}_o$ , non-glycosylated hemoglobin plus ( $\text{HbA}_1$ )) is particularly important with regard to that disease.

Hemoglobin  $\text{A}_{1c}$  is a glycohemoglobin with an amino acid structure which is identical to that of  $\text{HbA}_o$ ; the important difference is the presence of 1 - amino - 1 - deoxy - fructose attached to the 2,3 - diphosphoglycerate pocket to the N-terminal valine in the beta-chain of  $\text{HbA}_{1c}$ . The modification of  $\text{HbA}_o$  to  $\text{HbA}_{1c}$  is a continuous non-enzymatic post-translational process, the rate of which is a function of the blood glucose concentration. Glycosylation occurs as a two step process. Firstly, the open aldehydic form of glucose reacts with the terminal amino group of the beta-chains of Hb to form a Schiff base. Secondly, the Schiff base then undergoes an Amadori rearrangement to form  $\text{HbA}_{1c}$ . The intermediate Schiff base is unstable with a 60-fold greater tendency to dissociate (to the free sugar plus protein) than the stable ketoamine of  $\text{HbA}_{1c}$ . As only a small fraction of blood glucose is in the open aldehydic form (approximately 0.001%) and the rate of ketoamine formation is slow (although effectively irreversible), the formation of  $\text{HbA}_{1c}$  is an indication of long term blood glucose concentration. Over the 120 days life-span of the human red blood cell, the number of glycosylated Hb molecules increases proportionally to the mean blood glucose concentration. The relationship between the mean plasma glucose and  $\text{HbA}_{1c}$  concentrations is unique in that a single  $\text{HbA}_{1c}$  measurement provides a retrospective assessment of blood glucose control

over the preceding 6 to 8 weeks. It is generally accepted that HbA<sub>1c</sub> measurements are a very useful tool in monitoring diseases of carbohydrate metabolism, specifically diabetes mellitus. Diabetics have high long-term blood sugar levels and this is reflected in their HbA<sub>1c</sub> levels. Normal adults have about 3—6% of their total hemoglobin as HbA<sub>1c</sub> whereas the range in juvenile and maturity onset diabetics is 6—15% as HbA<sub>1c</sub>. A similar increase in HbA<sub>1c</sub> concentration has been noted in mice with genetic and chemically induced diabetes and in pancreatectomized dogs.

Among the several methods which exist for determining glycosylated Hb in blood, HbA<sub>1</sub> and in particular HbA<sub>1c</sub> measurements have now become a method of choice for monitoring the treatment of diabetics (L. Jovanovic et al., *American J. of Medicine* (1981) 70, 331; D. E. Goldstein et al., *Diabetes* (1982) 31, 70; K. H. Gabboy et al., *J. of Clinical Endocrinology and Metabolism* (1977) 44, 859; B. Gonen et al., *Diabetologia* (1978) 15, 1; C. M. Peterson, *Diabetes* (1982) 31, 1). Also, the following patent documents can be usefully mentioned: US—A—4,247,553; GB—A—1,580,318; US—A—4,222,836; US—4,372,747; 4,200,435; 4,341,635. These methods can be readily classified by the mechanism used to separate glycosylated Hb from non-glycosylated Hb. For instance ion-exchange chromatography was used early and is still the most common method (H. G. Kunkel et al., *Science* (1955) 122, 288). Although such ion-exchange technique is currently the only available method which specifically measures HbA<sub>1c</sub>, it has a number of limitations of which temperature and pH sensitivity are the most important. Ion-exchange also is subject to interferences as labile glycosylated Hb (pre-HbA<sub>1c</sub>) must be removed prior to assay and both fetal Hb (HbF) and Sickle Cell Hb (HbS) interfere with the results.

Other techniques involve agar gel electrophoresis (L. Menard et al., *Clinical Chemistry* (1980) 26, 1598), isoelectric focusing (K. M. Spicer et al., *Diabetes* (1978) 27, 384), colorimetry, e.g. with thiobarbituric acid (R. Fluckiger et al., *FEBS Letters* (1976) 71, 356) and affinity chromatography (V. Bouriotis et al., *Diabetologia* (1981) 21, 579). Only one type radioimmunoassay has been reported (J. David et al., *British J. of Haematology* (1978) 38, 329) which was slow (more than 3 days to work) and technically complex as requiring the preparations of radiolabelled HbA<sub>1c</sub>. Although the methods of the prior art have merits, there is still a need for methods providing quick results (less than about 15 minutes), requiring less skilled operators and less costly to be undertaken on a routine basis. Current art methods are slow (typically more than 1 hour results), technically complicated (require more than five pipetting manipulation steps) and unsuited to testing outside a laboratory environment. Further, present methods require that total hemoglobin be ascertained separately from the glycosylated factors and it would be desirable that both analytical data can be ascertained substantially together and correlated without delay.

The method of the present invention, as summarized in claim 9, remedies the inconvenience of prior art methods and further offers the advantage of directly relating, if desired, the percent of glycosylated factor or other hemoglobin factors to total hemoglobin.

The present method allows for the separate determination of (Hb) A<sub>1c</sub>, A<sub>1a</sub> or A<sub>1b</sub> provided antibodies specific to any of such species are available in purified form. Otherwise, using less specific antibodies, the present method allows for the combined determination of two or more blood factors taken together, i.e. all glycosylated Hb relative to total Hb for instance. Of course, the method also provides for the determination of blood factors other than the ones hereabove if corresponding reagents specific to said factors in complex formation reactions are available (e.g. HbF, HbS or other human hemoglobin variants).

The present invention does not concern the obtention or preparation of such specifically reactive complex moieties (monoclonal or polyclonal antibodies) which belong to the prior art but it concerns their use as coating materials in the preparation of the active wave guides to be contacted with the blood sample to be analyzed according to the invention.

The waveguides used in the present methods can be of many kinds and some have been disclosed in copending application EP—A—75353 together with the methods for coating the wave guides with the reactive antibodies selected.

In the present case, plate-like or fiber optic waveguides included as members of an analytical cuvette are preferably used, the coated surface of the wave guides being put into contact with the blood sample once the latter has been injected into the cuvette.

The optical technique used here relates, as discussed hereinabove, mainly to light absorption, i.e. there is an interaction of the evanescent component of the wave transported in the guide with the molecules, first in the ambient liquid (the depth of penetration the evanescent component exceeds somewhat the thickness of the antibody coating, which provides an instantaneous response) and, second, with the Hb-antibody complex which starts building up on the guide in forms of an additional layer due to the reaction of the blood factor to be determined with the specific complex moiety (antibody) previously coated on the guide surface. Although the depth of interaction of the evanescent light component is not substantially limited to the thickness of the layer of the complex, it has been surprisingly found that the optical response to that build-up is independent of the bulk absorption due to the blood itself and the two effects can be easily distinguished without sophisticated techniques for decoding the signals originating from one or the other effect.

Hb derivatives have characteristic absorption spectra dependent on their chemical state. Hence, any of the usual absorptiometric techniques are equally applicable for implementing the invention (L. Tentori et al., *Hemoglobin*, in *Methods in Enzymology* (1981), vol. 76, 707—732, Academic Press, New York). Included are the cyanomethemoglobin method and single or multi-wavelength absorptionmetric assays,

## EP 0 184 600 B1

preferably in the range 400 to 600 nm, specifically 400—420 nm and 550—600 nm. Also included are such isobestic point methods where the absorption by the Hb molecule is independent of the degree of oxygen saturation.

The invention and its present illustrative aspect will be better understood with help of the accompanying drawing.

Fig. 1a is a diagram explaining the propagation of totally reflected light in a medium (waveguide) of refractive index  $n_1$  greater than  $n_2$ , the refractive index of another medium (analyte) with which the wave guide is in contact.

Fig. 1b is appendent to Fig. 1a and schematically represents the penetration of the evanescent wave component in the rarer medium (analyte).

Fig. 2 is a schematic layout of a single waveguide apparatus for carrying out the method of the invention.

Fig. 3 is a schematic layout of another embodiment of an apparatus for carrying out the method of the invention.

Fig. 4a is a schematic top view of a detail of another embodiment of an analytical apparatus involving a double waveguide cell.

Fig. 4b is a schematic view of a variant of the embodiment of Fig. 4a.

Fig. 4c is a schematic view of still another embodiment.

Fig. 5 is a schematized representation of the phenomena occurring during an analysis according to the method of the invention.

Fig. 6 is a diagram showing response curves in performing an analysis according to one embodiment of the invention.

Fig. 7 is a diagram showing the variation of one parameter of the response curve as a function of the angle of incidence of the multiply reflected light beam travelling through the waveguide.

Fig. 8 is a diagram with a typical response curve in the analysis of HbA<sub>1c</sub> in the presence of hemoglobin.

Fig. 9 illustrates another type of analysis involving fluorescence.

As said before, when a light beam 1 strikes with an angle the interface between two transparent media  $n_1$  and  $n_2$  (Fig. 1a), striking from the medium  $n_1$  with the greater refractive index ( $n_1 > n_2$ ), total internal reflection occurs (N. J. Harrick, Internal Reflexion Spectroscopy, Wiley Interscience, New York (1967)) when the angle of reflection  $\theta$  is larger than a certain value  $\theta_c$  called the critical angle given by the equation:

$$\theta_c = \sin^{-1} (n_2/n_1) \quad (1)$$

The reflected beam is indicated by numeral 2. In this case the evanescent wave penetrates a distance ( $d_p$ ) of the order of a fraction of a wavelength beyond the reflecting surface into the rarer medium of refractive index  $n_2$ . According to Maxwell's equations a standing sinusoidal wave, perpendicular to the reflecting surface, is established in the denser medium (Fig. 1b). Although there is no net energy flow into the non absorbing, rarer medium, there is an evanescent, nonpropagating field 3 in that medium, the electric field amplitude (E) of which is largest at the surface interface ( $E_0$ ) and decays exponentially with distance (Z) from the surface according to:

$$E = E_0 \cdot \exp (-Z/d_p) \quad (2)$$

The depth of penetration ( $d_p$ ), defined as the distance required for the electric field amplitude to fall to  $\exp (-1)$  of its value at the surface, is given by:

$$d_p = \frac{\lambda/n_1}{2\pi(\sin^2 \theta - (n_2/n_1)^2)^{1/2}} \quad (3)$$

Starting from  $90^\circ$ , as  $\theta$  approaches  $\theta_c$ ,  $d_p$  becomes infinitely large, and at a fixed angle, increases with closer index matching (i.e., as  $n_2/n_1 \rightarrow 1$ ). Also, because  $d_p$  is proportional to wavelength, it is greater at longer wavelengths.

Thus, by an appropriate choice of the refractive index  $n_1$  of the transparent waveguide, of the incident angle, and of the wavelength, one can select a  $d_p$  to control optical interaction, either mainly with substances 4 close or at given distance from the interface and minimally with substances 5 beyond said distance or, with varying response ratios, with both 4 and 5. And this is precisely one of the key factors of the present invention, i.e. to have established that appropriate selection of said parameters ( $n_1$ ,  $\theta$  and  $\lambda$ ) provides optical conditions for measuring, at the same time, two independent parameters in the analyte. In the present embodiment, the denser medium can be constituted by a quartz microscope slide ( $n_1=1.54$ ) and the rarer medium is aqueous blood sample ( $n_2=1.34$ ) and  $\theta$  is controllably variable so that when  $\lambda$  is a selected visible wavelength,  $d_p$  can be varied from about 20 to 300 nm until optimal response is provided. Of course, other materials for the waveguide can be used with refractive indices other than 1.54.

Although single reflection systems can be used, one can enhance sensitivity (lower the limit of

## EP 0 184 600 B1

detection) by combining the evanescent wave principle with multiple internal reflections. The number of reflections (N) is a function of the length (L) and thickness (T) of waveguide and angle of incidence ( $\theta$ ):

$$N = L/T \cdot \cot \theta \quad (4)$$

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The microscope slide waveguides used in some of the experiments had an active length of 36 mm, thickness of 1 mm and the angle of incidence varied from about 60 to 75°. Thus the number of reflections on one side for a discrete light beam was approximately 6. Similarly, in another embodiment using a fibre optic waveguide, the latter had an active length of 64 mm, a thickness of 0.6 mm and, with the same angles of incidence the total number of reflections for a discrete light beam varied from about 30 to 40.

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As said before, the method of the invention can also rely on fluorescence effects. Fluorescence emission generated at a waveguide liquid interface can also be monitored at the waveguide output. As predicted by the theory of reciprocity and demonstrated with dye molecules in both monomolecular layers (Carnaglia and Mandel, *J. Optical Soc. of America* 63, 479 (1972)) and in monodispersed spheres (Lee et al., *Applied Optics* 18, 862 (1979)), fluorescence emission at a waveguide/liquid interface can be treated as an evanescent wave. In effect, excitation of fluorescence by an evanescent wave produces fluorescence emission with the characteristics of an evanescent wave and thus generates an internally reflected beam of fluorescent light. The direction of fluorescence emission of this form is mainly a function of the respective refractive index ratio and has the major characteristic (see the above Carnaglia reference) that photon emission has a distribution of "preferred" angular probability close to the critical angle ( $\theta_c$ ).

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In practical terms this means that fluorescence can be monitored at the output of the waveguide in the same optical plane as the excitation light. Theoretically, this has the advantages of concentrating the fluorescent emission intensity within a small angle; also, these fluorescent photons do not pass through the bulk of the solution and thus are not subject to major optical interference (e.g. absorption, scattering).

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This technique is disclosed in more detail in co-pending application EP—A—75353.

For the fluorescence measurements illustrating the present invention, the excitation wavelength was selected at 490 nm and fluorescence emission measured (wavelength greater than 510 nm) at the waveguide output by positioning cut-off filters before the photodetector element (KV 8.5; 50% transmittance at 550 nm. Schott Glass Works, Mainz, Germany).

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Fluorescence techniques allowing for the simultaneous determination of two or more parameters, for instance with multi-analyte waveguide systems have many applications in the field of clinical diagnostics, e.g. simultaneous measurement of the thyroid hormones  $T_4$  and  $T_3$ , the gonadotrophins LH and FSH, tumor markers such as AFP and CEA; also the whole gamut of cell surface antigen determination as applied to clinical microbiology.

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One embodiment of the apparatus used is schematically represented on Fig. 2 which shows a block diagram the major components; these components comprise a monochromator 9, a light source 6, a flow cell 7 with waveguide 8, and electronics with data-acquisition and processing microcomputer including a photomultiplier detector 10, a preamplifier 11, a computerized light source control system 12, a microprocessor 13, a printer 14, and a memory (floppy disc) 15.

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The light source 6 in this instance was a xenon flash lamp (E. G. & G., Salem, MA) and the monochromator was equipped with a concave holographic grating (Jobin-Yvon, Paris, France) to permit a resolution of 5 nm. The flash lamp operation was controlled by microcomputer 12. To inject the samples through an input 18 to the cell 7 a programmable automatic pipette (Microlab-P; Hamilton Bonaduz AG, Bonaduz, Switzerland) was preferably used. The optical component further included two mirrors  $M_1$  and  $M_2$  and two prisms 16 and 17. A photomultiplier tube of the detector 10 (R928; Hamamatsu, Tokyo, Japan) placed at the waveguide output monitored the change in light intensity directly. Signals from the photomultiplier tube were amplified (11), integrated during the flash time (12) and converted by a standard 12-bit analog/digital converter (not shown) into digital format. The in-house microcomputer 12 performed fast signal averaging, and all data were adjusted for variation in flash lamp intensity by reference to a photodiode 19 placed in the monochromator. The signals were transmitted to a microcomputer 13, preferably an Apple II model, for display and storage. Two different embodiments of waveguide systems were used:

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The analytical cell or cuvette illustrated on Fig. 2 as one embodiment, is based on a microscope slide waveguide system. The illustrated system shows the flow cell 7 whose bottom is actually the microscope slide 8. Tightness is ensured by a gasket 20; the slides 8 were placed in direct optical contact with two quarter-round silica prisms 16 and 17, preferably from Heraeus. Light was coupled into and out of the waveguide through these prisms by use of index matching oil, thereby removing the requirement for specially polished, optically flat waveguide faces. The prisms were designed to allow easy adjustment of the angle of incident light  $\theta$  (see Fig. 1a) and to avoid contact of light with the sealing gasket 20.

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The flow cell, machined from aluminium alloy, met the criterion of allowing rapid, bubble-free laminar flow along the light path. Its design also ensured rapid and accurate demounting and repositioning. We chose an aluminium alloy, although other metals are also suitable, e.g. brass, because of its good thermal conductivity, relative lack of reactivity with saline solution, and low optical reflectivity after being anodized matt black to avoid stray light effects. The gasket 20 was 0.5 mm thick medical grade silicone rubber and water tight under a constant sealing pressure of 2 kg/cm<sup>2</sup>. Including input 18 and exit 21 ports the total cell

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## EP 0 184 600 B1

volume was 1.8 ml, the volume directly above the waveguide was 0.66 ml (53×25×0.5 mm) and the volume above the light path was 0.29 ml (36×16×0.5 mm).

A second embodiment (see Fig. 3) is based on a fibre optic system. The fibre waveguide 31 was prepared from standard transmission optical fibres by first cutting them into 120 mm pieces, then removing the ethylene/tetrafluoroethylene coating and silicone cladding to expose an optically active surface area of 120 mm<sup>2</sup>. The ends of the fibres were bared and held within specially made stainless steel end-fittings 32 and 33 (7×3 mm internal diameter) for support and protection. The fibre flow cell 34 was an open-ended quartz tube (internal diameter 4 mm, length 80 mm) with input 35 and output 36 tubes added for inserting and removing sample. The fibres were set in place in the flow cell with silicone rubber plugs 37, 38. Light from source 39 was filtered (40) and focussed (41) with a lens onto the end of the fibre at a mean aperture angle of 68° (see Fig. 1); at the fibre output, light was refocussed by means of a lens 42 onto a photomultiplier tube 43.

The apparatus of which the essential optical components are schematized on Fig. 4a comprises a dual-waveguide cell 50 of which the main walls 51 and 52 constitute two independently energized elements that transport the exciting signal originating from a source 53 and whose inner walls, either bared, blocked or coated with a specific reactant, are in contact with the analyte solution contained in cell 50. The especially shaped light conductive walls of the cuvette can be provided by usual means, e.g. by molding with a transparent plastic, for instance lucite. These walls can be made from materials with the same or different refractive indices.

The beam of light 54 originating from source 53 is alternatively split into beams 55 and 56 by a rotating chopper mirror 57a, b. On Fig. 4a, this mirror 57 has been represented in two positions, i.e. one instant position corresponds to numeral 57a and another position (at about right angle to the first one) by numeral 57b. It can be readily seen that, depending on the position of the mirror 57, the beam 54 is either reflected into beam 55 or into beam 56. Thus, the light from the source 53 is alternatively injected in either portion 51 and 52 of the dual waveguide cell 50 by means of either one of a series of mirrors 58a, b, c and 59a, b and c, respectively. The output light, respectively 60 and 61, from either part of the waveguide is then collected on a detector 62.

The remaining components of this embodiment are not represented on the drawing as being known from the art and identical to the corresponding elements disclosed in connection with the embodiment of Fig. 2.

In a further embodiment (see Fig. 4b), the apparatus comprises a dual-waveguide cell 70 identical with the cell of the previous embodiment, i.e. having walls 71 and 72 acting as the two independent elements of the waveguide and operating similarly as will be seen.

The apparatus comprises a light source 73, the output of which is focussed on either side by means of lenses and mirrors, respectively 74 and 75, on the input side of guide elements 71 and 72, respectively. A chopper disk 76 with a window hole 77 acts to alternatively distribute the exciting light into elements 71 and 72. The output signals from the waveguide are then directed to a detector 78 by means of mirrors 79 and 80.

In both embodiments depicted on Figs. 4a and 4b, one of the waveguide elements (51, 71) is coated with an antibody specific to one component to be measured in the analyte by a complexation reaction (as disclosed hereintofores) while the second element (52, 72) is left uncoated. Herein, uncoated refers to a surface without antibody. However the protein adsorption sites on this surface are usually blocked by adsorbing a protein (e.g. BSA) to the surface. Therefore during analysis, the signal collected at the output of the uncoated area reflects the interaction of the exciting beam with the bulk of the analyte, i.e. it provides the desired information on the total hemoglobin in the sample. However, simultaneously, the signal emerging from the coated side of the guide provides the required information on the component being bound by the specific reactant coated on the inner surface of this side of the cell. This will be illustrated in more detail with reference to Example 4 in this application. Suffice to say now that this kind of waveguide system (dual type) allows to gather the two types of information from separate areas of the waveguide (i.e. the phenomena are no longer superimposed like in the earlier embodiment) which may provide more accuracy in the determinations.

A variant embodiment is represented on Fig. 4c. In this variant, a dual waveguide cell 90 of the same general configuration as the aforementioned cells 50 and 70 is used with the difference that ends 91a and 92a are actually made reflective, for instance by metallizing (silver) like with a mirror. Therefore, the other ends 91b and 92b, respectively, of the waveguide light conductive elements act simultaneously as input and output ends. This is illustrated by the paths of the exciting light beams provided by two sources 93 and 94 which are directed into ends 91b and 92b, respectively, after crossing beam-splitters 95 and 96, respectively. Thus, the light which penetrates through ends 91b and 92b travels through the guide first in a forward direction and then backwards after being reflected from ends 91a and 92a. This configuration enables that the interaction capacity of the exciting light with the analytes be actually doubled as compared with the previously disclosed embodiment. This variant further comprises a detector 97 for collecting the backward signals exiting from 91b and 92b and directed thereto by means of beam splitters 95 and 96 and a prism-like mirror 98. Sources 93 and 94 are alternately synchronized so that signal pulses exiting from the waveguide ends 91b and 92b do not simultaneously fall on the detector 97.

Fig. 5 is a schematic illustration at the molecular level of the phenomena taking place during analysis in a cell of the dual-waveguide type as disclosed previously. In Fig. 5 areas marked 51 and 52 correspond for



instance to the waveguide elements 51 and 52 depicted on Fig. 4a. The area intermediate between areas 51 and 52 represents schematically an analyte medium with species dissolved therein and reactants or species attached to the inside walls of elements 51 and 52. Element 51 is pictured to have deposited thereon antibodies 100 specific to HbA<sub>1c</sub>, entities labelled 101. Some of these HbA<sub>1c</sub> molecules are shown after  
 5 complexation with the specific antibody 100, others are still free. The other surface (i.e. the surface of element 52) is shown coated with blocking agents 102 (for instance, bovine serum albumin) said agents being intended to minimize the possible affinity of the bare wall to other species in solution, for instance HbA<sub>1c</sub> 103 and other proteins of any type 104.

Thus, during analysis, non specific binding of Hb to surface 52 is prevented (or at least strongly  
 10 minimized) which makes it possible to measure the bulk hemoglobin by the interaction of the evanescent wave component of the signal travelling in 52 with the analyte solution at depths beyond that of the blocking coating deposited on the surface.

In contrast, a complexation reaction occurs on surface 51 between the antibody molecules 100 coated thereon and the HbA<sub>1c</sub> (AG) molecules in the analyte solution. This reaction although rapid is not  
 15 instantaneous; therefore a layer of complex progressively builds up on surface 51 with consecutive corresponding interaction with the light component travelling in that element of the waveguide, this resulting in the production of response curves of the A or B type depicted in Fig. 6 (see the Examples that follow).

In order to practically carry out the tests, the microscope slides were cleaned by consecutive  
 20 immersion in concentrated sulfuric acid and distilled water, ethanol, and acetone, using standard slide-staining glassware. Fibres were cleaned in ethanol ultrasonically and, supported on glass rods, were immersed in the various antibody solutions. Antibodies were either physically adsorbed to the surface of the waveguides or covalently coupled. Adsorption was carried out by incubating cleaned waveguides with  
 25 solutions of antibody (5 mg of protein/ml of 0.05 mol/l Tris Hcc buffer, pH 7.0) for four hours. Unadsorbed proteins were washed away with saline and residual protein binding sites blocked by incubation of the antibody-coated waveguides with bovine serum albumin (1.0% by weight in Tris Buffer). The method of coupling was essentially that of Weetall, involving aminopropyltriethoxysilane APTS (Immobilized Biochemicals and Affinity Chromatography, R. B. Dunlop, Plenum Press, New York, p. 191—212) in an acid  
 30 aqueous silanization environment. (Immobilized Enzymes, Antigens, Antibodies and Peptides: Preparation and Chromatography, 1: Enzymology, H. A. Weetall, Marcel Dekker Inc. New York 1975, p. 1—48).

In general, we reacted waveguides with APTS (0.4 mol/l) for 3 hours at 80°C. We then heated the slides or the cuvette walls at 120°C and the fibres at 100°C for 2 hours, then let them soak in glutaraldehyde solution (0.5 mol/l) in phosphate buffer (0.1 mol/l, pH 6.8) for 90 min at ambient temperature. The  
 35 "activated" waveguides were then reacted with antiserum Ab (5 mg of protein per milliliter of phosphate buffer) for 24 hours at 4°C. After washing the antibody-coupled waveguides in phosphate buffer, we stored them at 4°C in isotonic saline (0.14 mol/l, containing sodium azide, 8 mmol/L). Measurements of protein (Anal. Biochem 51, 654—655 (1973)) before and after the coupling demonstrated protein uptakes of approximately 1 µg/cm<sup>2</sup> of quartz.

The following Examples illustrate the invention in more detail.

#### 40 Example 1

(to demonstrate the existence of two distinct optical phenomena

- i) interaction of the evanescent wave component with hemoglobin in bulk,
- ii) interaction of the evanescent wave component with the Ag/Ab complex under formation).

45 The apparatus used was that of embodiment of Fig. 2.

Preparation of standards with known solutions of hemoglobin.

Purified hemoglobin A (HbA) was obtained from Serva Feinbiochemica, Heidelberg, FRG. Bovine Serum Albumin (BSA) was from Sigma Chemical Co., St. Louis, Mo, USA. All chemicals for buffers and solvents were Analar or Reagent Grade from Merck, Darmstadt, FRG, or BDH, Poole, Dorset, UK. Rabbit  
 50 antiserum to human HbA was purchased from Dako, Copenhagen, Denmark.

Waveguides were fused-silica microscope slides (Suprasil 1.75 mm×25 mm×1 mm) from Heraeus Quarzschmelze GmbH, Hanau, FRG.

Slides were cleaned by consecutive immersion (10 min each) in concentrated sulphuric acid, distilled water, ethanol and acetone. Antibody was coated to the surface by incubating the cleaned slides for 1 hour  
 55 in a solution of anti-HbA diluted five-fold in phosphate buffered saline (PBS; 0.1 mol/L phosphate, pH 7.4, 0.9% (w/v) NaCl). Following rinsing with distilled water, remaining protein binding sites were blocked by incubation for 1 hour with 1% (w/v) BSA in PBS. Slides were then rinsed in distilled water and stored at 4°C in isotonic saline prior to use.

Slides were fixed in conformity to the first embodiment illustrated on Fig. 2 in a manner which allowed  
 60 light to be coupled into the slides at different angles θ. The flow cell 7 was fixed to the surface via a 0.5 mm silastic gasket 20 and bubbles purged from the system by pumping assay buffer (PBS+5.0% (w/v) BSA) through the cell. Standard Hb solutions (1.0, 0.5, 0.1, 0.05 mg/ml) were made up in assay buffer to give a final protein concentration of 5 mg total protein/ml.

The assay procedure was initiated by injecting 3.5 ml of standard Hb solution into the cell after  
 65 establishing a base-line signal. The wavelength of the input beam was selected by adjusting the



## EP 0 184 600 B1

monochromator at 410 nm and the reaction was monitored by the reduction in intensity at 410 nm. The angle  $\theta$  was first selected at random above  $66^\circ$  (the critical angle). A value of about  $67^\circ$  was used in the test reported below.

Illustrated by Fig. 6 are the antibody-binding curves obtained with the 1.0 (curve A) and 0.1 mg/ml (curve B) Hb standards, using successive antibody-coated slides. After stabilising the base-line, the standards were injected at  $t_0$  and an immediate fall ( $I_A, I_B$ ) in transmission (arbitrary units) was followed by a slower but still rapid binding event which continued over the next 10 minutes. The initial fall was due to free hemoglobin molecules optically absorbing within the  $D_p$  range of the evanescent wave (see Fig. 1). Note that at this early stage the complex layer is starting to form; therefore the evanescent wave component extends quite significantly beyond the initial Ab coating and is free to interact with the bulk solution. The subsequent slower change in signal of rate K and magnitude, respectively,  $M_A$  and  $M_B$  at time  $t_1$  was due to antibody-binding of Hb at the surface. In the absence of antibody the signal changed minimally after the initial immediate fall. This was shown in a control experiment not shown on the drawing using no Ab coating.

Then the cell was washed with assay buffer ( $t_1$ ) which removed all unbound materials. The residual absolute change in signal (WA, WB) is related to dose as indicated in the table below.

Absolute change in signal

	Test	Replicate	Mean
Dose Hb (mg/ml)	1	2	
0.1	-4.6%	-4.3%	-4.3%
1.0	-14.9%	-12.5%	-13.7%

The standard curves A and B were usable as templates for the determination of hemoglobin in unknown samples of blood. Similarly reproducible information could be gathered from measured values  $M_A$  and  $M_B$  provided unknown samples were measured after a constant time  $t_1$ .

### Example 2

#### Optimization of the incident angle ( $\theta$ ) vs sensitivity

With an antibody-coated slide in place, Hb standard solution (1 mg/ml) was injected into the flow cell. After 10 minutes reaction the unbound materials were washed out of the cell with assay buffer. Bound materials were monitored by the reduction in transmission at 410 nm. The effect of incident angle of light was investigated by varying the angle ( $\theta$ ) from  $64^\circ$  to  $78^\circ$ . The critical angle ( $\theta_c$ ) is  $66^\circ$ . The results were plotted as the % transmission decrease (=sensitivity) vs angle of incidence (Fig. 7). It can be seen that with this system measurement of antibody-binding of Hb is possible with angles between  $66$  at  $70^\circ$ , with an optimum near to  $68^\circ$ . Larger angle shave too small a penetration depth in this case although they may suit analytical system of a different kind; smaller angles result in refraction not reflection. Angles between  $66$  and  $68^\circ$  are less suitable presumably because the depth of penetration of the evanescent wave component is too great.

### Example 3

#### Preparation of more refined standard curves

Hb standard solutions were incubated with separate antibody-coated slides and the reaction monitored using  $\theta$ =about  $68^\circ$  as the optimal angle. The results expressed as % transmission, show a dose-response relationship:

Standard Hb (mg/ml)	% Transmission
1.0	87.2
0.5	94.0
0.1	94.4
0.05	95.3
0	100

The minimum detection limit of this system is about 0.1 mg/ml or 0.1 g/l. Normal adult HbA values are 135—175 g/l, normal HbA<sub>1c</sub> levels are 4—9 g/l, thus this method can be mixed with  $\times 10$ — $\times 100$  dilution of normal samples with adequate sensitivity.

## EP 0 184 600 B1

### Example 4

Measurement of hemoglobin in the presence of foreign hemoglobin.

Solution samples were prepared based on avian hemoglobin (pigeon) and containing variable proportions of human hemoglobin to be measured. The total of both hemoglobins was always 5 mg/ml and the proportions of human hemoglobin are given in the Table below. A dual waveguide of the type shown in Figs. 4a and 4b was used, one of the surfaces (e.g. 51) being coated with antibody to human IgG. The other surface (52) was blocked with bovine serum albumin as usual.

Upon doing the measurements a sharp dip (I) corresponding to 75.3% transmission was observed in all cases; then the further drop in transmission (M) (cf. with Example 1 and Fig. 6) was recorded after an interval of 10 min. In the case of the sample containing only avian hemoglobin, no further change during the 10 min interval was observed. The results are summarized below.

	Human Hb in avian Hb (%)	Transmission (%) (after 10 min)	M
15	0	75.3	0
	1	74.9	0.4
20	2	74.4	0.9
	10	72.0	3.3
25	20	68.3	7.0

Thus the value recorded for the first initial dip I can be correlated with the total hemoglobin present while the values (M) observed after the 10 min reaction period and corresponding to the binding of the human hemoglobin factor to the antibody coated on surface 51 can be correlated with the human hemoglobin content of the sample and its ratio to total hemoglobin. Standard curves were made from the above data by recording on an automatic recorder coupled to the apparatus used in this Example. Such curves were thereafter used as comparative data for determining unknown mixtures of human hemoglobin in avian hemoglobin.

### Example 5

Measurement of glycosylated hemoglobin (HbA<sub>1c</sub>) in the presence of hemoglobin

Standard glycosylated Hb (HbA<sub>1c</sub>) was prepared from pooled heparinized whole blood by cation-exchange chromatography (L. A. Trivelli et al., New England J. of Medicine 284 (1971), 353), using Bio-Rex 70 resin (Bio-Rad, Richmond, Ca. USA). The purified HbA<sub>1c</sub> was then used to prepare standard samples by recombining it in varying known amounts with blood free from the glycosylated hemoglobin. The concentrations of HbA<sub>1c</sub> relative to total hemoglobin in the samples varied from 1 to 20% by weight and the total Hb concentration was of the order of 150 g/l.

An analytical apparatus with cuvettes involving a dual waveguide as illustrated on Fig. 4b was used for the determinations; the inner surface of one side of the cuvette was plated with antibody specific to HbA<sub>1c</sub> while the surface of the opposite side was left free. The content of each cell (a fresh one was used for successively testing each standard) was about 1 ml and 0.1 ml of the standard to be measured with about 0.9 ml of PBS were pipetted therein. Fig. 8 depicts one of the titration curves obtained after 15 min incubation time (with the 20% HbA<sub>1c</sub> sample), the upper curve (nearly flat) being that recorded with the uncoated part of the guide and the lower curve showing the response of the antibody coated part of the waveguide.

The results of the analysis of the various standards are also gathered in the table below.

	Standard sol. under test (% HbA <sub>1c</sub> )	% Transmission in waveguide		Difference M (%)
55		Uncoated side	Coated side	
	0	56.1	55.8	0.3
	1	55.5	54.7	0.8
60	5	55.7	50.2	5.5
	10	58.0	49.2	8.8
65	20	54.9	42.4	12.5

## EP 0 184 600 B1

The difference of 0.3% for the zero HbA<sub>1c</sub> sample may indicate some degree of residual affinity of the HbA<sub>1c</sub> specific antibody for the deglycosylated blood medium. This factor is however considered negligible under practical analytical conditions.

It should also be noted that the % transmission in the uncoated part of the waveguide was not constant from one cell to the other seeming to indicate that the method is not suitable for accurately determining total Hb. However it is not necessary in this instance to measure total Hb, but only to relate the signals from the uncoated and coated sides. Secondly, it is difficult to maintain a degree of constancy in manually fabricating a series of cuvettes such that each will enable full reproducibility of absolute measurements without initial calibration of the equipment. Undoubtedly, when cuvettes are manufactured industrially by molding on a large scale, this disadvantage is overcome.

### Example 6

The simultaneous determination of human IgG and human serum albumin (HSA) by fluorescence type assay

The dual waveguide system as in the previous Example was used, a cut-off filter to block the incident radiation at 490 nm and pass the fluorescent signal at 520 nm being inserted on the light path before detector 78. The exciting light was generated by a monochromator of the type (9) disclosed in connection with Fig. 2.

One wall surface (A) of the cuvette serving as the dual-waveguide was coated with sheep raised antiserum against IgG. This was effected by adsorption according to usual means using a diluted solution of antiserum (γ-chain specific; Sapu, Carluke, Scotland; dilution by volume 1/400). The oppositely facing wall (B) of the cuvette was coated by the same technique with sheep antiserum against HSA (final dilution 1/100 by volume) obtained from the same source.

Then, mixed combination standard solutions were prepared by dissolving together human IgG (Serva Biochemicals) and HSA (UCB-Bioproducts, Brussels, Belgium). Concentrations of reagents in the standards were 0.1; 1.0; 10.0 and 100.0 µg/ml. The solution buffer used as the standard medium was phosphate buffer 0.05 mole/l (pH 7.4); 0.9% NaCl (W/v); 0.05% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (W/v); Tween 20 (Sigma) 0.1% (v/v) and 2% (v/v) normal sheep serum (SAPU).

The test disclosed in this experiment was based on making a "sandwich" type assay i.e. the cuvettes were contacted with the standards and incubation was carried out for a determined period to ensure sufficient binding of the antigens on the respective specific antibodies attached to surfaces (A) and (B). This incubation time was an accurately measured 10 min period in the course of which the amount of overall bound antigen was in proportion to its concentration in the standard. Tests against blanks (zero % of antigen reagents) were run identically.

Thereafter, the cells were emptied and rinsed of all unbound materials and a combined solution of second antibodies to the antigens attached to the waveguide surface was added. This combination solution contained 1/40 (v/v) buffer diluted rabbit anti-HSA and rabbit anti-IgG (obtained from Dako Immunoglobulins) labelled with fluorescein (fluorescein isothiocyanate, FITC was used as the actual marker according to usual means).

Once the fluorescent labelled mixed antibody solution was added, an instantaneous fluorescence rise was observed at the output of the waveguide followed by a slower rate dependent signal (see Fig. 9) whose height after a given period was in proportion to the standard concentrations in IgG and HSA taken independently. After decoding, the signals components originating from surfaces (A) and (B) were displayed separately and the results are gathered in the following table.

Concentration of IgG and HSA in standards (µg/ml)	Response from surface A (arbitrary units)			Response from surface B (arbitrary units)		
	Test	Repeat	Average	Test	Repeat	Average
0.1	1	2	1.5	—1	2	1
1.0	27	24	25	44	44	44
10.0	64	61	62	60	56	58
100.0	160	170	165	140	135	137

Fig. 9 shows graphically for components A and B the situation from zero to 15 min in the case of the 1 µg/l (dashed line) and 10 µg/l (mixed line) standards. The full line represents blanks.

As in the previous Example, samples with unknown concentrations of IgG and HSA were run identically and ascertained by comparing with the standards.

## Claims

1. A method for ascertaining at least one parameter of species in a liquid analyte, in which the evanescent component of a fully reflected light signal interacts, at the interface between said analyte and a waveguide (50, 70, 90) of said signal, with a monomolecular layer of a complex constituted by one of said species and a reagent (100, 102) specific thereto attached to the guide surface and/or the bulk of the analyte, thus providing a light energy output representative of said parameters, the method including collecting said output energy and processing it opto-electronically into readout data corresponding to said parameters, characterized by either:
  - a1) predetermining the angle of reflection of said light signal in the waveguide for allowing said evanescent wave component to penetrate beyond said complex layer into the bulk of the solution to a depth sufficient to be responsive to both said complex layer and said bulk of the analyte and provide processable output energy on a parameter representative of a first species (101) in said complex layer and a parameter representative of a second species (103, 104) in the solution, or
  - a2) using two or more distinct areas (51, 71, 91; 52, 72, 92) of the waveguide, one first area (51, 71, 91) retaining a first reagent attached thereto to provide a complex layer of a first species to be determined, whereby said interaction is only responsive to said complex layer and the output is only representative of a parameter of this first species, and a second or further area (52, 72, 92) the latter being either:
    - a21) bare or blocked, whereby said interaction is responsive to the bulk of the analyte and representative of a parameter of a second species dissolved therein or
    - a22) coated with a second or further reagent specific of a second or further species thus providing a layer of a second or further complex to which said interaction is responsive and the output is representative of another parameter, the latter pertaining to said second or further species,
  - b) processing the output consisting of a mixed signal and splitting it into components specific of each parameter of interest,
  - c) expressing said components as separate data, and
  - d) ascertaining said parameters by correlating the displayed data with standard data obtained from standard solutions in which the corresponding parameters are known.
2. The method of claim 1, wherein the excitation light in the waveguide is transmitted by multiple reflections therein and wherein the interaction of this signal with the analyte involves some degree of absorption, and/or scattering, and/or the generation of fluorescence.
3. The method of claim 2, wherein the waveguide consists of an optical slide or fiber (51, 52; 71, 72; 91, 92) and a layer of complex forms during analysis at the surface thereof thus providing a time dependent response on said first species and instant response on said second species.
4. The method of claim 2, wherein said two or more distinct areas (51, 52; 71, 72; 91, 92) of the waveguide are energized by the same incident light signal.
5. The method of claim 2, wherein said two or more distinct areas of the waveguide each involve separately illuminated waveguide elements.
6. The method of claim 5, wherein said waveguide is constituted by an analytical cuvette (50; 70; 90) whose oppositely facing walls operate as independent separately illuminated elements.
7. The method of any of claims 3 to 5 wherein said mixed signal collected at the output comprises components at the same or different wavelengths.
8. The method of claim 5, wherein the illumination of said distinct areas is done in alternation.
9. The method of claim 1 adapted for substantially simultaneously determining total hemoglobin Hb in a blood sample and, selectively at will, further hemoglobin factors or derivatives thereof such as glycosylated hemoglobins Ag relative to said total hemoglobin, this method comprising the following steps:
  - a) Coating at least part of the surface of an optical waveguide system (50, 70, 90) of a refractive index  $n_1$  greater than that  $n_2$  of the sample to be analyzed with one or more coatings of complexing reactants Ab each coating being of a reactant specific to that factor or derivative of hemoglobin Ag and capable of building up a layer of said complex upon reaction with said Ag;
  - b) Illuminating said waveguide at an input end (91b; 92b) with a beam of light (54) and collecting the exiting light (60, 61) at an output end, said beam being transmitted along said guide by a multiple internal reflexion mechanism at an angle  $\theta$  such that the effective range of action outside the guide of an evanescent light component associated with said beam transmission exceeds that of said complex layer;
  - c) Bringing into contact said blood sample to be analyzed and said illuminated waveguide whereby, on one hand, part of the light travelling in the guide is initially absorbed by interaction of the evanescent wave component with the hemoglobin of the bulk sample, this resulting in an instantaneous sharp dip I in the exiting light from said output end and, on the second hand, an immuno-type reaction develops between said glycosylated hemoglobin or other factor to be determined in said blood sample and corresponding Ab coated on the guide with consecutive build-up of said complex layer, such developing resulting in a relatively slower change to said exiting light, said relatively slower change being due to the interaction of said evanescent wave component with said complex layer in formation;
  - d) Observing, measuring and/or recording said sudden optical absorption dip I occurring to the

## EP 0 184 600 B1

transmitted light collected from said output end, said measured dip being quantitatively related with the concentration of the total hemoglobin Hb of the sample;

e) Observing, measuring and/or recording said relatively slower change, the magnitude M and rate K of which quantitatively relates to the amount of glycosylated hemoglobin or other factor Ag in the sample;

5 f) Effecting the required computations e.g. electronically, to express the results obtained from the values of I and M or K in terms of concentration of Hb and/or ratio of Ag to Hb in the sample.

10 10. The method of claim 9, wherein steps (d) and (e) are both effected on the signal resulting from the interaction of the incident light and the analyte at the area of the waveguide bearing the antibody Ab coated thereon.

11. The method of claim 9, wherein step (d) is effected on the component of the output signal resulting from said interaction on a part of the waveguide not coated with the antibody Ab.

12. The method of claim 11, wherein there is used a dual type waveguide (50, 70, 90), one portion of which (51, 71, 91) is coated with antibody Ab and another portion of which (52, 72, 92) is not coated with antibody.

13. The method of claim 12, wherein said other portion is coated with a blocking protein for minimizing the possible deposition of hemoglobin on the waveguide uncoated surface area.

14. The method of claim 12, which comprises using as a dual waveguide an analytical cuvette (50, 70, 90) whose main opposite walls are light conductive and made of a transparent material of suitable refractive index  $n_1$  greater than that  $n_2$  of the blood sample, this material serving as the waveguide.

### Patentansprüche

1. Verfahren zum Nachweis mindestens eines Parameters einer Substanz in einem flüssigen Analyten, bei dem die verschwindende Komponente eines vollständig reflektierten Lichtsignals an der Grenzfläche zwischen dem Analyten und einem Wellenleiter (50, 70, 90) des Signals mit einer monomolekularen Schicht aus einem Komplex zusammenwirkt, der durch eine der genannten Substanzen und ein dafür spezifisches Reagens (100, 102) gebildet und an die Leiteroberfläche und/oder die Analytmasse gebunden ist, um so einen Lichtenergieendwert zu schaffen, der für diese Parameter repräsentativ ist, wobei das Verfahren des Auffangen dieser Ausgangsenergie und ihre optoelektronische Bearbeitung zu diesen Parametern entsprechenden ablesbaren Werten umfaßt, dadurch gekennzeichnet, daß entweder:

a1) der Reflexionswinkel des Lichtsignals in dem Wellenleiter vorbestimmt wird, um zu ermöglichen, daß die verschwindende Wellenkomponente nach außerhalb der Komplexschicht in die Lösungsmasse eindringen kann bis in eine Tiefe, die ausreichend ist, um sowohl auf die Komplexschicht als auch auf die Analytmasse anzusprechend und eine bearbeitbare Endergie auf einen Parameter, der für eine erste Substanz (101) in dieser Komplexschicht repräsentativ ist, und einen Parameter, der für eine zweite Substanz (103, 104) in der Lösung repräsentativ ist, zu schaffen, oder

a2) zwei oder mehrere getrennte Bereiche (51, 71, 91; 51, 72, 92) des Wellenleiters verwendet werden, von denen ein erster Bereich (51, 71, 91) ein erstes daran gebundenes Reagens zum Schaffen einer Komplexschicht einer ersten zu bestimmenden Substanz enthält, wodurch diese Wechselwirkung nur auf diese Komplexschicht anspricht und der Endwert nur für einen Parameter dieser ersten Substanz repräsentativ ist, und ein zweiter oder weiterer Bereich (52, 72, 92) entweder:

a21) freiliegend oder blockiert ist, wodurch diese Wechselwirkung auf die Analytmasse anspricht und für einen Parameter einer darin gelösten zweiten Substanz repräsentativ ist oder

a22) mit einem zweiten oder weiteren Reagens beschichtet ist, das für eine zweite oder weitere Substanz spezifisch ist, um so eine Schicht aus einem zweiten oder weiteren Komplex zu bilden, auf welchen diese Wechselwirkung anspricht, und der Endwert für einen weiteren Parameter repräsentativ ist, der der zweiten oder weiteren Substanz zugehörig ist,

b) der aus einem gemischten Signal bestehende Endwert bearbeitet und in die für die jeweils interessierenden spezifischen Parameter spezifischen Komponenten zerlegt wird,

c) diese Komponenten als separate Werte ausgedrückt und

d) diese Parameter bestimmten werden, indem die angezeigten Werte zu Standardwerten in Beziehung gesetzt werden, die aus Standardlösungen mit bekannten entsprechenden Parametern erhalten sind.

2. Verfahren nach Anspruch 1, worin das erregende Licht in dem Wellenleiter durch mehrfache Reflexionen darin weitergeleitet wird und wobei die Wechselwirkung dieses Signals mit dem Analyten ein gewisses Ausmaß an Absorption und/oder Streuung und/oder Fluoreszenzbildung einschließt.

3. Verfahren nach Anspruch 2, wobei der Wellenleiter aus einem optischen Leiter oder einer Faser (51, 52; 71, 72; 91, 92) besteht und während der Analyse an seiner Oberfläche eine Komplexschicht bildet, um so eine zeitabhängige Reaktion auf die erste Substanz und eine sofortige Reaktion auf die zweite Substanz zu schaffen.

4. Verfahren nach Anspruch 2, wobei die zwei oder mehr getrennten Bereiche (51, 52; 71, 72; 91, 92) des Wellenleiters durch dasselbe einfallende Lichtsignal angeregt werden.

5. Verfahren nach Anspruch 2, wobei die zwei oder mehr getrennten Bereiche des Wellenleiters jeweils separat beleuchtete Wellenleiterelemente umfassen.

6. Verfahren nach Anspruch 5, wobei der Wellenleiter durch eine Analyseküvette (50; 70; 90) gebildet

## EP 0 184 600 B1

ist, deren entgegengesetzt aneinanderliegenden Wände als unabhängige, separat beleuchtete Elemente arbeiten.

7. Verfahren nach einem der Ansprüche 3 bis 5, wobei das am Ausgang aufgefangene gemischte Signal Komponenten gleicher oder unterschiedlicher Wellenlängen enthält.

5 8. Verfahren nach Anspruch 5, wobei die Beleuchtung der getrennten Bereiche abwechselnd geschieht.

9. Verfahren nach Anspruch 1, angepaßt für die weitgehend gleichzeitige Bestimmung von Gesamthämoglobin Hb in einer Blutprobe und wahlweise weiterer Hämoglobinfaktoren oder ihrer Derivate, wie glykosyliertes Hämoglobin Ag, bezogen auf Gesamthämoglobin, wobei das Verfahren aus den folgenden Stufen besteht:

10 a) Beschichten mindestens eines Teils eines optischen Wellenleitersystems (50, 70, 90) mit einem Brechungsindex  $n_1$  von größer als  $n_2$  der zu analysierenden Probe mit einer oder mehreren Beschichtungen von komplexbildenden Reagentien Ab, wobei jede Beschichtung ein Reagens ist, das für den Faktor oder das Derivat des Hämoglobins Ag spezifisch und befähigt ist, eine Schicht dieses Komplexes nach Reaktion mit diesem Ag aufzubauen;

15 b) Beleuchten dieses Wellenleiters an einem Eingangsende (91b; 92b) mit einem Lichtstrahl (54), und Auffangen des erregten Lichts (60, 61) an einem Ausgangsende, wobei der Strahl entlang des Leiters durch einen inneren Mehrfachreflexionsmechanismus bei einem Winkel  $\theta$  in der Weise weitergeleitet wird, daß der effektive Aktionsbereich außerhalb des Leiters einer von dieser Lichtstrahlweiterleitung begleiteten verschwindenden Lichtkomponente den der Komplexschicht überschreitet;

20 c) Kontaktieren der zu analysierenden Blutprobe und des beleuchteten Wellenleiters, wodurch einerseits ein Teil des in dem Leiter wandernden Lichts zu Beginn durch Wechselwirkung der verschwindenden Wellenkomponente mit dem Hämoglobin der Massenprobe absorbiert wird, was zu einer sofortigen scharfen Neigung I in dem von dem Ausgangsende erregten Licht führt, und sich andererseits eine immunotypische Reaktion zwischen dem glykosylierten Hämoglobin oder einem  
25 anderem zu bestimmenden Faktor in der Blutprobe und dem entsprechenden auf dem Leiter aufgetragenen Ab entwickelt mit danach folgendem Aufbau der Komplexschicht, wobei diese Entwicklung zu einer relativ geringeren Veränderung auf das erregte Licht führt, wobei die relativ geringere Veränderung eine Folge der Wechselwirkung der verschwindenden Wellenleiterkomponente mit der in Ausbildung befindlichen Komplexschicht ist;

30 d) Beobachten, Messen und/oder Aufzeichnen der bei dem weitergeleiteten, am Ausgangsende gesammelten Licht auftretenden plötzlichen optischen Absorptionsneigung I, wobei die gemessene Neigung in quantitativer Beziehung zu der Konzentration an Gesamthämoglobin Hb der Probe steht;

35 e) Beobachten, Messen und/oder Aufzeichnen der relativ geringeren Veränderung, deren Größe M und Verhältnis K in quantitativer Beziehung zu der Menge des glykosylierten Hämoglobins oder eines anderen Faktors Ag in der Probe steht;

f) Ausführen der erforderlichen Berechnungen, z.B. elektronisch, zum Ausdrücken der Ergebnisse, die aus den Werten von I und M oder K, ausgedrückt durch die Konzentration an Hb und/oder das Verhältnis von Ag zu Hb in der Probe, erhalten sind.

40 10. Verfahren nach Anspruch 9, wobei die Stufen (d) und (e) beide aufgrund des Signals bewirkt werden, das durch Wechselwirkung des einfallenden Lichts mit dem Analyten in dem Bereich des den Antikörper Ab als Beschichtung tragenden Wellenleiters erhalten wird.

11. Verfahren nach Anspruch 9, wobei die Stufe (d) aufgrund der Komponente des Ausgangssignals bewirkt wird, das durch Wechselwirkung auf einem Teil des Wellenleiters, der nicht mit dem Antikörper Ab beschichtet ist, erhalten wird.

45 12. Verfahren nach Anspruch 11, wobei ein Wellenleiter (50, 70, 90) vom Dualtyp verwendet wird, von dem ein Teil (51, 71, 91) mit einem Antikörper Ab beschichtet und ein anderer Teil (52, 72, 92) nicht mit dem Antikörper beschichtet ist.

50 13. Verfahren nach Anspruch 12, wobei der andere Teil zum Minimieren der möglichen Abscheidung von Hämoglobin auf der unbeschichteten Wellenleiteroberfläche mit einem blockierenden Protein beschichtet ist.

14. Verfahren nach Anspruch 12, bei dem als Dual-Wellenleiter eine Analyseküvette (50, 70, 90) verwendet wird, deren gegenüberliegende Hauptwände leichtleitend und aus durchsichtigem Material mit geeignetem Brechungsindex  $n_1$ , der größer ist als  $n_2$  der Blutprobe, hergestellt ist, wobei dieses Material als Wellenleiter dient.

### Revendications

1. Procédé pour mesurer au moins un des paramètres analytiques de certaines substances contenues dans un analyte liquide, suivant lequel il se produit, à la surface d'un guide d'onde (50, 70, 90) en contact  
60 avec cet analyte, une interaction entre le composant évanescent d'un signal lumineux se propageant par réflexion totale dans ce guide d'onde et une couche monomoléculaire d'un complexe constitué par l'une de ces substances et un réactif (100, 102) spécifique de celle-ci lié à la surface du liquide, et/ou la masse de l'électrolyte, de manière que soit émis un signal lumineux dont l'énergie correspond à la valeur desdits paramètres, ce procédé consistant à recueillir cette énergie émise et à la traiter optoélectroniquement de  
65 manière à en obtenir des résultats de lecture correspondant auxdits paramètres, caractérisé en ce que, soit:

## EP 0 184 600 B1

- a1) on ajuste l'angle de réflexion dudit signal lumineux dans le guide d'onde de façon à ce que ledit composant d'onde évanescent puisse pénétrer dans la masse de la solution à une profondeur suffisante au delà de la couche de complexe pour réagir aussi bien avec celle-ci qu'avec la couche de complexe et fournir un signal de sortie correspondant simultanément à un paramètre relatif à une première substance (101) de ladite couche de complexe et à un paramètre relatif à une seconde substance (103, 104) de la solution, soit:
- a2) on met en oeuvre deux ou plusieurs zones différentes (51, 71, 91; 52, 72, 92) du guide d'onde, une première zone (51, 71, 91) où est fixé un premier réactif destiné à donner une couche d'un complexe formé par réaction avec une première des substances à déterminer, ladite interaction ne se produisant qu'avec ladite couche de complexe et le signal de sortie correspondant n'étant, représentatif que d'un paramètre de cette première substance, et une seconde zone (52, 72, 92), ou d'autres encore, celle-ci étant:
- a21) libre ou recouverte d'un inhibiteur, de manière que l'interaction se produise dans la masse de la solution d'analyte et corresponde à l'un des paramètres d'une seconde substance dissoute dans celle-ci, ou
- a22) recouverte d'un second réactif spécifique d'une seconde substance, ou encore d'autres réactifs spécifiques d'autres substances, destiné à fournir une couche d'un second complexe, ou d'autres encore, ce complexe réagissant avec le signal lumineux de manière que l'énergie émise corresponde à un autre paramètre, relatif, celui-ci, à ladite seconde substance, ou à d'autres substances,
- b) on effectue le traitement de l'énergie émise, laquelle consiste en un signal mixte, et on la divise en ses composants, chacun d'eux spécifiant l'un des paramètres recherchés,
- c) on exprime la valeur de chaque composant sous forme de résultats distincts, et
- d) on mesure lesdits paramètres en comparant les résultats affichés avec des données de référence obtenues à partir de solutions de référence dont les paramètres correspondants sont connus.
2. Procédé suivant la revendication 1, dans lequel le signal lumineux d'excitation se propage par réflexion multiple dans le guide d'onde et dans lequel l'interaction de ce signal avec l'analyte provoque l'absorption, et/ou la diffusion partielle du signal, et/ou une émission de fluorescence.
3. Procédé suivant la revendication 2, dans lequel le guide d'onde consiste en une plaquette ou fibre de verre optique (51, 52; 71, 72; 91, 92) et suivant lequel une couche d'un complexe se forme à la surface de celle-ci au cours de l'analyse, de manière que le signal de réponse correspondant à la première substance croisse au cours du temps, alors que celui qui représente la seconde substance apparaisse instantanément.
4. Procédé suivant la revendication 2, dans lequel l'énergie lumineuse parcourant lesdites deux zones distinctes (51, 52; 71, 72; 91, 92) du guide d'onde, ou d'autres encore, est fournie par un même signal lumineux incident.
5. Procédé suivant la revendication 2, dans lequel lesdites deux zones distinctes du guide d'onde, ou d'autres encore, consistent chacune en un élément distinct de celui-ci illuminé séparément.
6. Procédé suivant la revendication 5, dans lequel ledit guide d'onde consiste en une cuvette d'analyse (50, 70, 90) dont les parois se faisant face fonctionnent comme éléments illuminés séparément.
7. Procédé suivant une quelconque des revendications 3, à 5, dans lequel ledit signal mixte recueilli à la sortie comporte des composants de même ou différentes longueurs d'ondes.
8. Procédé suivant la revendication 5, dans lequel on éclipse alternativement lesdites zones distinctes du guide d'onde.
9. Procédé suivant la revendication 1, adapté pour mesurer pratiquement simultanément l'hémoglobine totale Hb d'un échantillon sanguin et, sélectivement suivant les besoins, d'autres facteurs d'hémoglobine ou leurs dérivés, tels que les hémoglobines glycosylées Ag, relativement à ladite hémoglobine totale, ce procédé comprenant les étapes suivantes:
- a) on revêt au moins une partie de la surface d'un guide d'onde optique (50, 70, 90) d'indice de réfraction  $n_1$  supérieur à l'indice  $n_2$  d'un échantillon à analyser avec une ou plusieurs couches de réactifs de complexation Ab, chaque revêtement se rapportant à un réactif spécifique de l'un des facteurs d'hémoglobine ou ses dérivés Ag et capable de former une couche de ce complexe par réaction avec ledit Ag;
- b) on éclaire ledit guide d'onde par une extrémité d'entrée (91b, 92b) au moyen d'un faisceau lumineux (54) et on recueille la lumière réfléchie (60, 61) à une extrémité de sortie, ledit faisceau se propageant dans le guide d'onde par réflexion interne multiple sous un angle  $\theta$  de valeur telle que la partie active du composant d'onde évanescente relatif audit faisceau s'étende au delà de la couche dudit complexe;
- c) on met en contact ledit échantillon sanguin à analyser et ledit guide d'onde illuminé, ce qui fait que, d'une part une portion du flux lumineux parcourant le guide d'onde est absorbée tout d'abord par interaction du composant d'onde évanescent et de l'hémoglobine de la masse de l'échantillon, d'où une chute nette immédiate I du signal lumineux émergeant de ladite sortie, et d'autre part se déroule une réaction de type immunologique entre l'hémoglobine glycosylée ou un autre facteur à mesurer dans l'échantillon sanguin et le réactif Ab lié au guide ce qui conduit à la formation progressive de ladite couche de complexe, cet événement conduisant à une variation relativement plus lente dudit signal de sortie, cette variation relativement plus lente provenant de l'interaction du composant d'onde évanescent et de ladite couche de complexe en formation;
- d) on observe, mesure et/ou enregistre ladite soudaine augmentation d'absorption I de la lumière transmise et recueillie à ladite extrémité de sortie, la grandeur mesurée de cette variation correspondant quantitativement avec l'hémoglobine totale Hb de l'échantillon;
- e) on observe, mesure et/ou enregistre ladite variation relativement plus lente dont la grandeur M et la



## EP 0 184 600 B1

vitesse de variation K correspondent quantitativement à la quantité d'hémoglobine glycosylée, ou un autre facteur Ag, dans l'échantillon;

f) on effectue les calculs nécessaires, par exemple par traitement électronique, de manière que les résultats tirés des valeurs de I et de M ou K soient exprimés en terme de concentration de Hb et/ou de rapports de concentration de Ag à Hb dans l'échantillon.

10. Procédé suivant la revendication 9, dans laquelle on applique les étapes (d) et (e) aux signaux qui résultent de l'interaction du faisceau incident et de l'analyte dans la zone du guide d'onde revêtue de la couche d'anticorps Ab.

11. Procédé suivant la revendication 9, dans lequel on applique l'étape (d) au composant du signal de sortie qui résulte de ladite interaction dans la zone du guide d'onde non revêtue de l'anticorps Ab.

12. Procédé suivant la revendication 11, dans lequel on emploie un guide d'onde double (50, 70, 90) dont une portion (51, 71, 91) est revêtue d'anticorps et dont une autre portion (52, 72, 92) n'est pas revêtue d'anticorps.

13. Procédé suivant la revendication 12, dans lequel cette autre portion est revêtue d'une protéine d'inhibition de manière à éviter autant que possible que l'hémoglobine ne se dépose à la surface de la zone non revêtue de guide d'onde.

14. Procédé suivant la revendication 12, dans lequel on utilise comme guide d'onde double une cuvette d'analyse (50, 70, 90) dont les parois principales opposées conduisent la lumière et sont faites d'un matériau transparent convenable d'indice de réfraction  $n_1$  plus élevé que celui,  $n_2$ , de l'échantillon sanguin, ce matériau fonctionnant comme guide d'onde.

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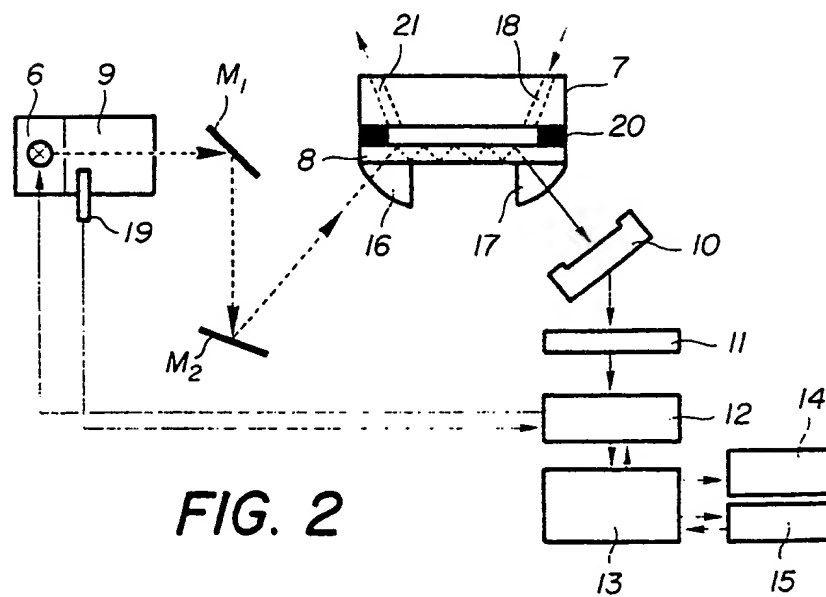
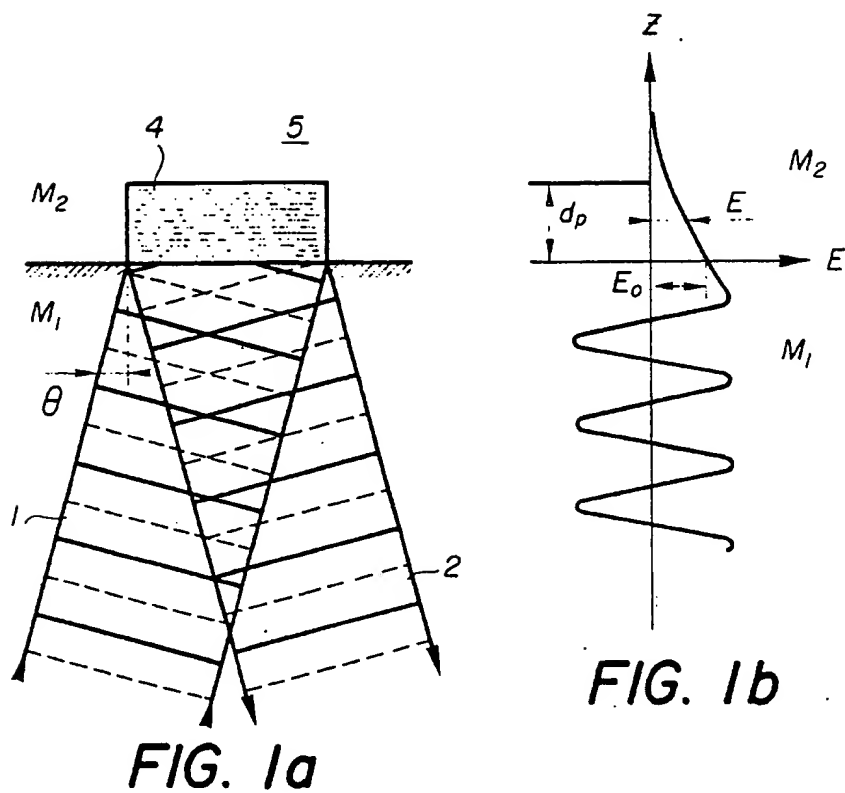
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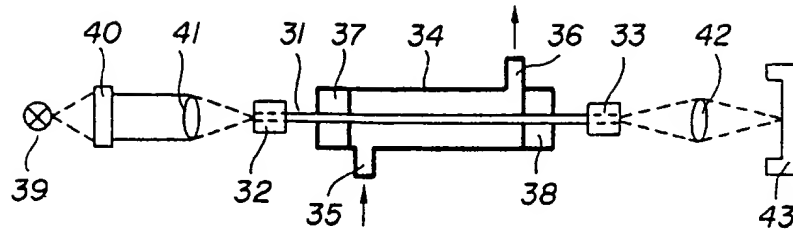


FIG. 3

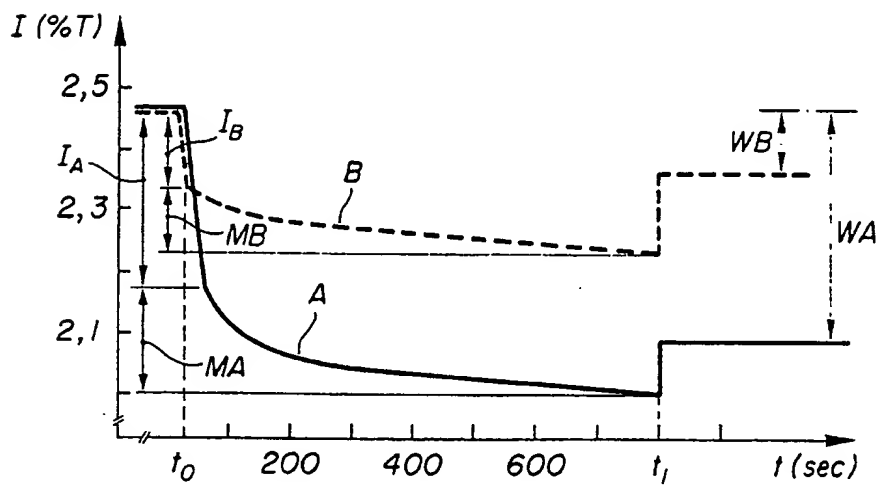


FIG. 6

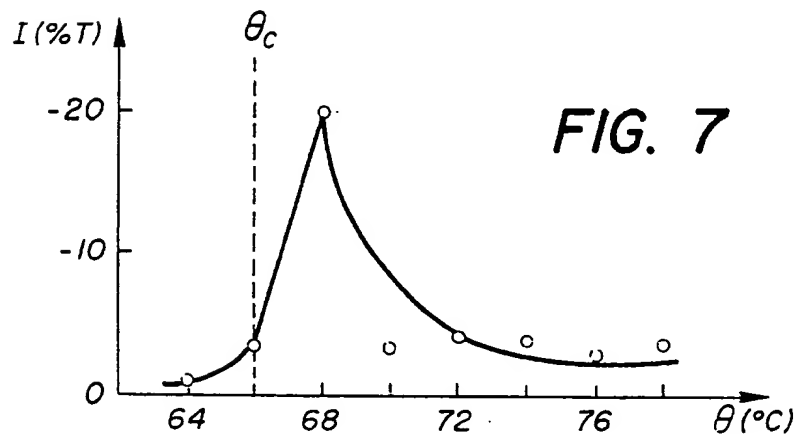
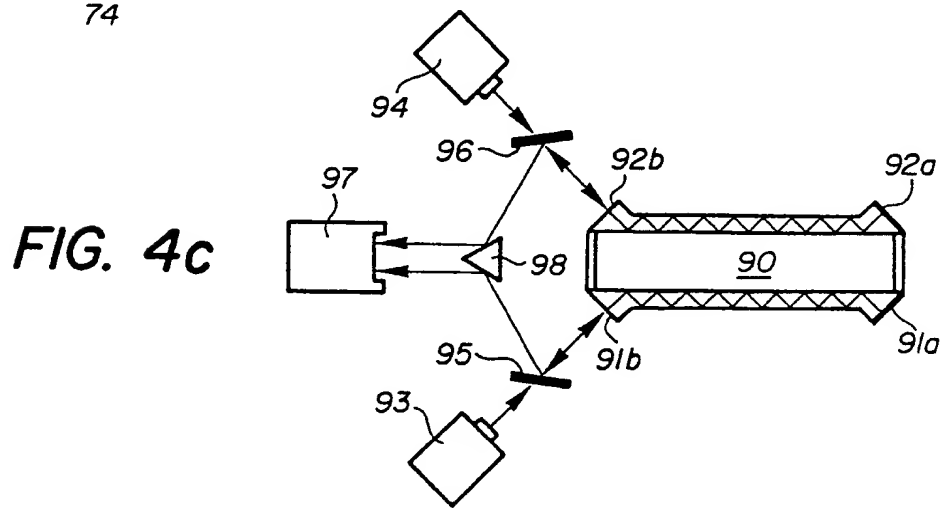
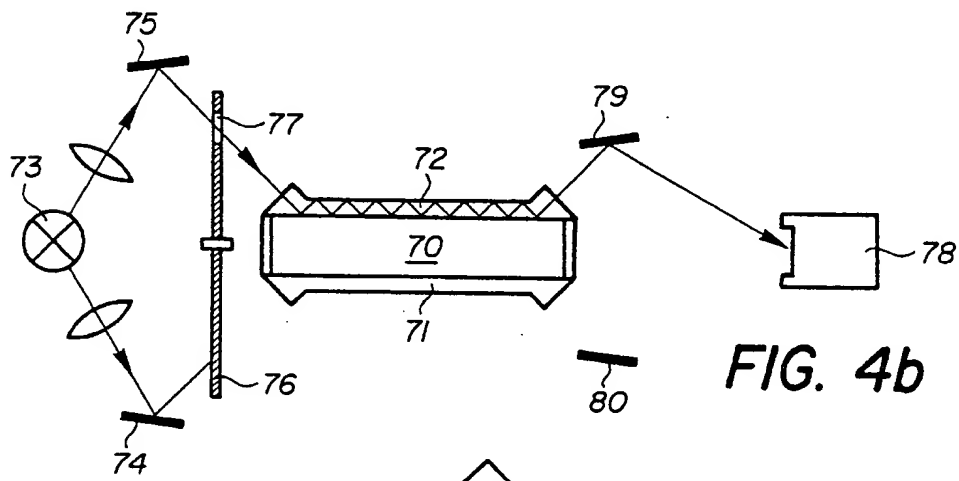
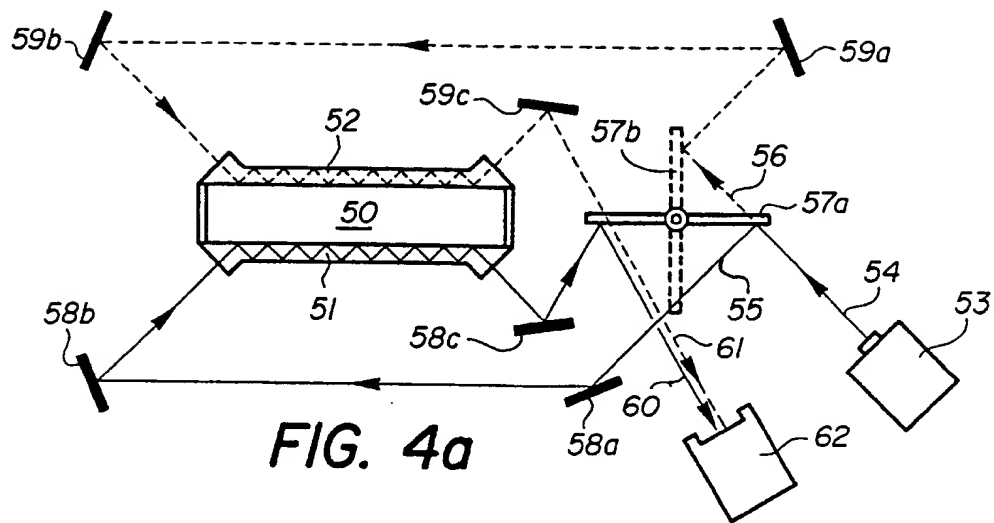


FIG. 7



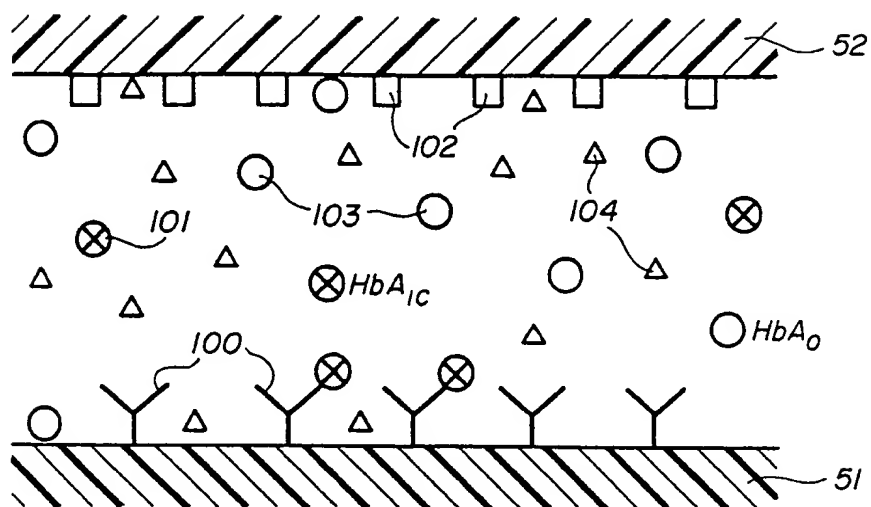


FIG. 5

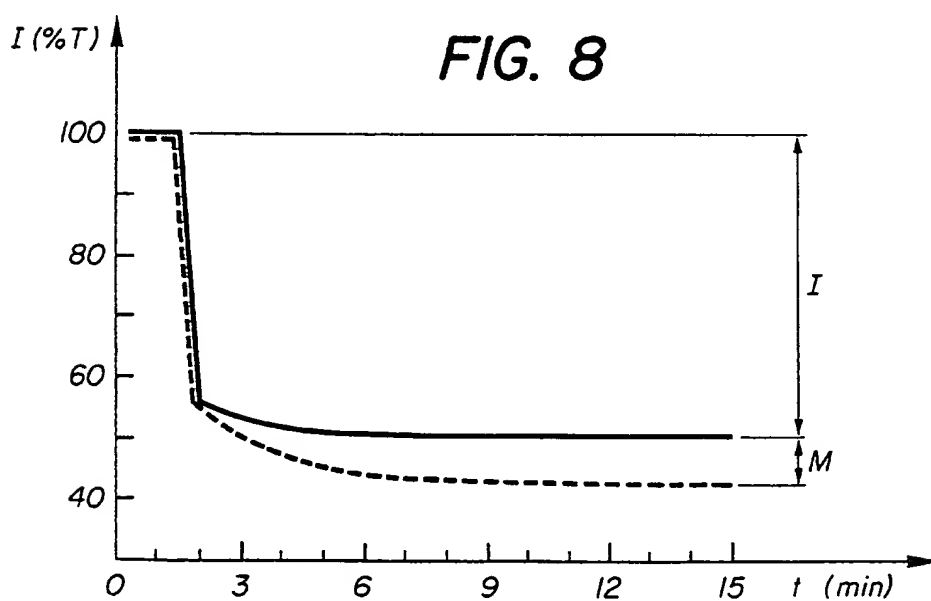


FIG. 8

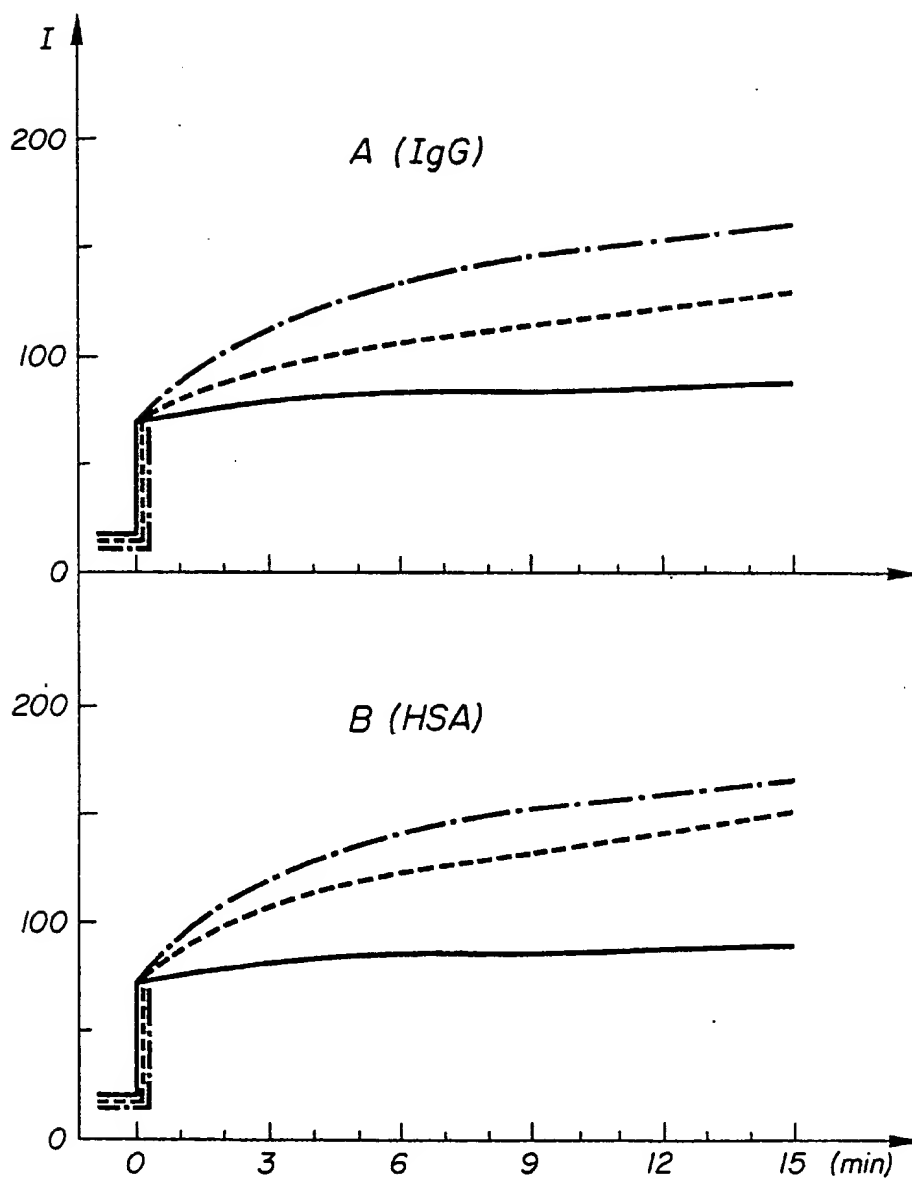


FIG. 9

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